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Flow Cytometry Used to Assess Genetic Damage in Frogs from Farm Ponds

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Flow cytometry (FC) is a laboratory method used to detect genetic damage induced by environmental contaminants and other stressors in animals, including amphibians. We tested FC methods on three species of ranid frogs collected from farm ponds and natural wetlands in southeastern Minnesota. We compared FC metrics for *Rana clamitans* between ponds with direct exposure to agricultural contaminants and reference (unexposed) ponds. Concentrations of atrazine in water from our farm ponds ranged from 0.04 to 0.55 ppb. We found that *R. clamitans* from exposed ponds had DNA content similar to frogs from unexposed ponds. Pond-averaged C-values (a measure of DNA content) ranged from 6.53 to 7.08 for *R. pipiens* (n = 13), 6.55 to 6.60 for *R. clamitans* (n = 40) and 6.74 for *R. palustris* (n = 5). Among all species, the mean sample CVs ranged from 1.91 (*R. palustris*) to 6.31 (*R. pipiens*). Deformities were observed in only 2 of 796 individuals among all species and occurred in both reference and exposed ponds. Although we did not detect evidence of DNA damage associated with agriculture in our study, we demonstrated the potential of FC for screening amphibian populations for genetic damage. Metrics from a variety of amphibian species and locations as well as laboratory studies are needed to further assess the value of FC for monitoring amphibian genetic integrity in contaminated sites.

INDEX DESCRIPTORS: flow cytometry, *Rana clamitans,* farm pond, genetic damage.

Global amphibian population declines have prompted increased interest in monitoring amphibian populations and identifying factors associated with these declines (Wake 1991, Alford et al. 2001, Storfer 2003). Amphibian populations with exposure to environmental toxicants are a high priority for monitoring and assessment (Little et al. 2003). Pesticides and fertilizers associated with agriculture (Berrill et al. 1997, Sparling et al. 2001, Knutson et al. 2004, Relyea 2005) can be detrimental to amphibian populations. Many known toxicants have mutagenic effects on amphibians in laboratory studies (Harfenist et al. 1989). Toxicants can induce clastogenic formation of micronuclei, leading to abnormal DNA content (Fernandez et al. 1993, Krauter 1993). Amphibians that breed in farm ponds are exposed to a wide range of agricultural chemicals that may be associated with genetic effects (Lowcock et al. 1997) as well as deformities (Bridges et al. 2004).

Flow cytometry (FC) is a laboratory method used to detect genetic damage induced by environmental contaminants and other stressors in animals, including amphibians (Bickham et al. 1988, Lamb et al. 1991, Fernandez et al. 1993). FC monitors multiple cellular characteristics, estimates cellular DNA content, and detects small changes in DNA caused by exposure to environmental contaminants (Dallas and Evans 1990). FC can also be used to screen for abnormal DNA profiles, such as aneuploid mosaicism, a chromosomal condition associated with exposure to pesticides (Lowcock et al. 1997).

FC has rarely been used to assess genetic damage in amphibians. FC was measured in amphibians (*Rana clamitans*) in a study in Quebec (Bonin et al. 1997, Lowcock et al. 1997, Murphy et al. 1997). Genetic effects were discovered in otherwise apparently healthy animals living in association with agricultural fields exposed to pesticides. Another study testing the genotoxic potential of atrazine showed that it was not associated with increased nuclear heterogeneity at levels of exposure representing conditions found in the agricultural Midwestern U.S. (Freeman and Rayburn 2004). FC metrics from a variety of amphibian species and locations as well as laboratory studies are needed to further assess the value of the approach for monitoring amphibian genetic integrity in contaminated sites.

We tested FC methods on three species of ranid frogs collected during deformity assessments of farm ponds and natural wetlands from an agricultural landscape in southeastern Minnesota. We compare FC metrics for *R. clamitans* between ponds exposed to agricultural contaminants and reference (unexposed) ponds. We expected that blood from frogs developing in exposed ponds would exhibit more abnormal DNA profiles than blood from frogs developing in reference ponds.

METHODS

Our study area included two counties in southeastern Minnesota (Houston and Winona Counties) during June and July, 2000. These counties are dominated by agricultural land uses, mainly corn and soybean production. We sampled amphibian metamorphs from exposed and reference farm ponds in the study area. Our exposed ponds were constructed farm ponds surrounded by agricultural row crops (corn or soybeans) or pastures grazed by domestic livestock. Reference ponds were natural wetlands, generally marshes and oxbows of river floodplains, or farm ponds surrounded by grassland free from grazing by domestic livestock. Exposed ponds received higher inputs of fertilizers, pesticides, and animal wastes than the reference ponds; assays of nitrogen, phosphorus, and atrazine in the ponds support these differences (Knutson et al. 2002, 2004). Herbicides are applied to 97% of the corn acreage in the 18 top-producing corn states in the United States, including Minnesota (Hunst and Gowse 2001).

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The herbicide, atrazine is one of the most pervasive agricultural chemicals found in surface waters in the United States. In a study of midwestern reservoirs, 92% were found to be contaminated with atrazine; concentrations were generally < 5 ppb (Solomon et al. 1996). Nitrate is another pervasive agricultural chemical in the study area, one which can have adverse effects on aquatic life (Mueller et al. 1997).

We examined amphibians from four reference and five exposed ponds in the field for deformities. Amphibian deformity assessments were conducted when amphibians were late-stage metamorphs (Gosner Stage 42 or later). Deformity assessments consisted of examining as many as 100 individual metamorphs for physical malformations. Up to 10 random metamorphs, including any deformed individuals, from each pond were collected live for necropsy and blood collection for FC analysis.

We generally followed the laboratory methods of Lowcock et al. (1997) to estimate FC metrics. Blood samples for FC analysis were collected in the laboratory from 58 metamorphs representing three species: *R. clamitans* (n = 40), *R. pipiens* (n = 13), and *R. palustris* (n = 5). Individuals were euthanized with methane tricaine sulfonate prior to blood sampling (MS-222; Argent Laboratories, Redmond, Washington); two 10-μL samples of auricular blood were collected with 40-μL heparinized capillary tubes. Each sample was resuspended in 1.5-mL cryovials containing 200-μL freezing solution (0.25 *M* sucrose, 0.04 *M* trisodium citrate, 5% dimethyl sulfoxide; pH 7.61) and manually agitated. Blood specimens were flash frozen in liquid nitrogen and stored at −70° C until analysis. In addition, metamorphs were necropsied to determine their overall health and examined for *Ribeiroia* and other parasites.

We also collected blood from *Xenopus laevis* for purposes of calibrating our DNA test. Accurate estimation of DNA content for the target species requires calibration with the DNA content of an internal reference (Tiersch et al. 1989). A reference specimen of known DNA content obtained from *X. laevis* (6.3 pg of DNA/diploid nucleus, certified free of potential mutagens) was used as an internal control in every sample (Xenopus Express, Homosassa, Florida). A healthy *X. laevis* was euthanized as previously described and 4 mL of auricular blood were gathered with a 10-mL syringe and transferred to 80 mL of freezing solution. After manual agitation, the solution was allowed to incubate at room temperature for 1 min to allow tissue and clotted blood to settle out of solution. After incubation, 210-μL aliquots of reference blood were transferred into 1.5-mL cryovials and flash frozen in liquid nitrogen. Reference blood was stored at −70° C until needed.

Before sample preparation, ribonuclease stock solution (2 mg/mL) was prepared by adding 20 mg of Ribonuclease A (Sigma Chemical, St. Louis, Missouri) to 10 mL of autoclaved deionized water. One hundred mL of stock staining solution (API: 0.01 *M* Trizma Base, 0.01 *M* NaCl, 0.1% NP-40; pH 7.62) was combined with 300 μL of ribonuclease solution and 5 mg of propidium iodide (Sigma Chemical, St. Louis, Missouri). The solution was brought to a final volume of 140 mL using distilled water. The API staining solution was covered to prevent exposure to light and was stored at 4° C until needed. Before each run of samples, 300 μL of fresh ribonuclease stock solution was added to the API staining solution.

Samples for FC analysis were thawed and 200 μL of *Xenopus laevis* reference blood was added to each target sample. Samples were gently vortexed and immediately placed in an ice bath. The API staining solution (750 μL) was added to each sample, gently mixed, returned to the ice bath, and reincubated in the dark for 2 hours. After incubation, each sample was transferred to a 12 × 75 mm culture tube by filtration through 53 μm nylon mesh to remove clumped cells or tissue debris.

After incubation and filtration, samples were analyzed for DNA content using a FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, California). Before data acquisition, the linearity and alignment were calibrated with DNA Quality Control Particles (Becton-Dickinson Immunocytometry Systems). For each target sample, 20,000 stained nuclei were collected. Propidium iodide, when excited by the argon laser (488 nm), emits fluorescent light over the range of 550–650 nm that is detected by a photomultiplier tube (FL2) within the flow cytometer. The FL2-Width vs. FL2-Area dot plots were used to detect and differentiate erythrocytes from debris, and FL2-A histograms were used to determine DNA content. Data were analyzed using CellQuest software (Becton-Dickinson Immunocytometry Systems). Triplicate analysis was performed on 10% of the samples to assess intra-assay variation; the relative SD, defined as the SD divided by the mean, of the triplicate sample runs was 1.63%.

The C-value (pg of DNA/haploid nucleus) and an estimated sample half-peak coefficient of variation (CV) were calculated for every sample. The C-value was calculated from the following equation (Lowcock et al. 1997):

$$C_t = C_r P_t / P_r \quad (1)$$

where C_t is the C-value of the target species, C_r is the C-value of the internal reference, P_t is the peak channel of the target species, and P_r is the peak channel of the internal reference. The estimated sample CV is defined as the SD of the target peak divided by the mean channel of the target peak, multiplied by 100, and was calculated by the analysis software. The C-values and CVs were averaged among replicate FC analyses of the same individual to obtain a mean value for every specimen. Histograms with the target species' DNA profile were analyzed for aneuploid mosaicism.

All statistical comparisons were performed using the CV values for *R. clamitans*. The association between CV and pond exposure status was estimated using a general linear mixed model and restricted maximum likelihood (Littell et al. 1996). Ponds, as the experimental units, were treated as random effects. This method was also used to estimate the correlation among CVs measured on frogs obtained from a common pond.

RESULTS AND DISCUSSION

Pond-averaged C-values ranged from 6.53 to 7.08 for *R. pipiens* (n = 13), 6.55 to 6.60 for *R. clamitans* (n = 40) and 6.74 for *R. palustris* (n = 5; Table 1). Among all species, the mean CVs ranged from 1.91 (SE = 0.07; *R. palustris*) to 6.31 (SE = 0.23; *R. pipiens*). *R. clamitans* from exposed ponds (n = 3) had similar DNA content (mean CV) compared to frogs from unexposed ponds (n = 2; β = 0.01, SE = 0.24, t-test, df = 3, P = 0.98). Residuals were normal (Anderson-Darling test, P > 0.25). We had high power (≥90%) to detect an increase of ≥0.5 CV units from a reference mean of 3.0 CV using as few as three ponds in each of two pond types, reference and exposed (Thomas and Krebs 1997, Zar 1999). The estimated within-pond correlation among mean CV was low (r = 0.08, restricted likelihood ratio test, p = 0.48), indicating that mean CV's for *R. clamitans* from different ponds were similar to those from the same pond.

Deformities were observed in only 2 of 796 individuals among all species and occurred in both reference and exposed ponds. The CV's of the deformed frogs were low, 2.15 and 3.85 for the exposed and reference ponds, respectively. *R. clamitans* individuals from three ponds—two exposed and one reference pond—were found to contain the parasite *Ribeiroia ondatrae* (Table 1). *R. ondatrae* were found in the limb bud region of the deformed individuals, indicating that the deformities were most likely associated with parasite loads (Johnson et al. 1999, Johnson et al. 2002). *R. pipiens* from an exposed pond

Table 1. Amphibian deformity, DNA, and parasite statistics for farm ponds in southeastern Minnesota, 2000.

Species	Collection date	Minnesota township	Exp. group[a]	n (field)[b]	Deform. (field)[b]	n (lab)[c]	Mean C-value[d]	Mean CV (SE)[e]	Aneu-ploid peaks[f]	Rib. present[g]
R. clamitans	6-15-2000	Altura	Exp	25	1	9	6.55	3.02 (0.30)	1	1
R. clamitans	6-28-2000	Brownsville	Exp	30	0	4	6.57	2.87 (0.33)	0	2
R. clamitans	5-25-2000	Utica	Exp	276	0	8	6.57	3.28 (0.14)	0	0
R. clamitans	6-15-2000	Utica	Ref	102	1	10	6.57	2.83 (0.15)	0	1
R. clamitans	5-25-2000	Lewiston	Ref	42	0	9	6.60	3.32 (0.14)	0	0
R. palustris	7-19-2000	Eitzen	Ref	37	0	5	6.74	1.91 (0.07)	0	0
R. pipiens	7-12-2000	Houston	Ref	36	0	7	7.08	2.88 (0.28)	2	0
R. pipiens	7-06-2000	Sheldon	Exp	107	0	3	6.53	2.83 (0.37)	0	0
R. pipiens	7-05-2000	Caledonia	Exp	91	0	3	6.91	6.31 (0.23)	3	0

[a]Exp = ponds exposed to agricultural contaminants and Ref = unexposed ponds
[b]Number of individuals deformed (Def.) and total examined (n) in the field deformity assessment
[c]Number of individuals used in laboratory FC analyses and examined for parasites
[d]Average DNA weight (pg of DNA/haploid nucleus)
[e]Average coefficient of variation (CV; SD of the target peak, divided by the mean of the target peak channel, multiplied by 100)
[f]Number of individuals showing an aneuploid peak with flow cytometry (FC) analysis
[g]Number of individuals with the parasite *Ribeiroia ondatrae*

had aneuploid peaks and the highest mean CV among all ponds, but no deformities (Table 1).

Aneuploid mosaicism was observed in 10% of the specimens analyzed, with the highest number of abnormal profiles occurring in *R. pipiens* (Table 1). However, aneuploid mosaicism is not always indicative of genetic damage and may arise through spontaneous variation in amphibian populations (Lowcock and Licht 1990). In summary, we found little evidence that agricultural land uses surrounding breeding ponds were associated with genetic damage or higher malformation rates.

Concentrations of atrazine in water from farm ponds in our study area ranged from 0.04 to 0.55 ppb (μg/L, J. Elder, U.S. Geological Survey, Water Resources, Middleton, Wisconsin, unpublished data, June 2001). These concentrations are orders of magnitude lower than those shown to be lethal (47.6 mg/L, *R. pipiens* larvae, 96-h LC50) in the laboratory (Howe et al. 1998). The literature is mixed regarding atrazine effects on amphibians at concentrations commonly found in the environment. Allran and Karasov (2000, 2001) did not find alterations in survival or behavior of several native frog species. Sullivan and Spence (2003) found that higher atrazine exposure increased time to metamorphosis and decreased weight, length, and hematocrit of *X. laevis* tadpoles. Hayes et al. (2002, 2003) reported that concentrations as low as 0.1 ppb of atrazine induced endocrine disruption in *R. pipiens* and *X. laevis*. Christin et al. (2004) found that a mixture of pesticides, including atrazine, altered immune function in *X. laevis* and *R. pipiens*. These effects may go unnoticed in many field studies.

We expected that *R. clamitans*, a primarily aquatic species that overwinters as a tadpole in southeastern Minnesota, would show effects of aquatic contamination because of its long exposure to the breeding pond. Our findings contrast with a study of *R. clamitans* in Quebec, Canada, where researchers using FC demonstrated that adult and juvenile (metamorphs) frogs exposed to agricultural contaminants from potato fields had more DNA alterations than frogs from ponds adjacent to cornfields and control ponds; only juveniles had more deformities (Bonin et al. 1997). However, our C-values for *R. clamitans* (6.55 to 6.60 pg of DNA/haploid nucleus, Table 1) were within the range of values observed in the Canadian study (Bonin et al. 1997).

Although we did not detect evidence of DNA damage associated with agriculture in our study, we demonstrated the potential of FC for screening amphibian populations for genetic damage. FC metrics from a variety of amphibian species and locations are needed as baseline information, for comparison with other locations, and for establishing associations with a variety of environmental factors. FC metrics for most North American amphibian species are currently nearly absent from the literature. With refinement, FC is potentially nonlethal (Sparling et al. 2000, p.715) and may be used in concert with other screening tools when environmental contamination is known or suspected. For example, FC could be used to assess the effects on amphibians of agricultural pesticides individually or in combination at concentrations commonly encountered in the field. Laboratory exposure studies can assess what levels of pesticides are likely to induce genetic effects before field studies are undertaken. We suggest that amphibians with known genetic damage (positive controls) be included in future studies involving FC to provide a benchmark for comparisons with exposed and unexposed populations.

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